PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12Q 1/68, A61K 39/00, 39/395, C07K 13/00, 15/28

A1

(11) International Publication Number: WO 94/23067

(43) International Publication Date: 13 October 1994 (13.10.94)

(21) International Application Number: PCT/US94/03507

(22) International Filing Date: 31 March 1994 (31.03.94)

(30) Priority Data:
040,800 31 March 1993 (31.03.93) US

(71) Applicant: ABBOTT LABORATORIES [US/US]; CHAD 0377/AP6D-2, One Abbott Park Road, Abbott Park, IL 60064-3500 (US).

(72) Inventors: REILLY, Edward, B.; 1136 Winners Circle, Libertyville, IL 60048 (US). EISEN, Herman, N.; Nine Homestead Street, Waban, MA 02168 (US). TSOMIDES, Theodore; P.O. Box 1856, Cambridge, MA 02238 (US).

(74) Agents: GORMAN, Edward, H., Jr. et al.; Abbott Laboratories, CHAD 0377/AP6D-2, One Abbott Park Road, Abbott Park, IL 60064-3500 (US).

(81) Designated States: AU, CA, JP, KR, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: TUMOR-ASSOCIATED ANTIGENS RECOGNIZED BY T CELLS AND THE USES OF THESE ANTIGENS

(57) Abstract

This invention relates to the field of tumor immunology, and specifically to a novel family of melanoma-specific antigens recognized by T cells. These antigens, like all T cell epitopes, are in the form of small peptides associated with major histocompatibility complex antigens on the cell surface. Methods and materials for purification and sequence determination of these peptides are presented. Also presented are applications for their use in cancer diagnostics and therapy.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

Tumor-Associated Antigens Recognized by T Cells and the Uses of these Antigens

Field of the Invention

This invention relates to the field of tumor immunology, and specifically to a novel family of melanoma-specific antigens recognized by T cells.

Background of the Invention

Specific antigen recognition by T cells is based on the binding of the T cell receptor (TCR) to a complex composed of antigenic peptide and a major histocompatibility complex (MHC) molecule [Bjorkman, P. J., et al. (1987) Nature 329: 512]. $CD8^+$ cytotoxic T lymphocytes (CTL) recognize cell surface components formed by class I MHC glycoproteins and antigenic peptides. Antigenic peptides arise by cleavage of endogenously synthesized proteins that are bound by newly synthesized class I molecules in the endoplasmic reticulum and subsequently transported to the cell surface [Townsend, A. and Bodmer, H. (1989) Ann. Rev. Immunol. 7: 601]. These CTL epitopes are believed to be short peptides of about 7-12 amino acids that bind to a cleft on the surface of class I MHC molecules [Van Bleck, G. M. (1990) Nature 348: 213; Falk, K., et al. (1991) Nature 351: 290; and Udaka, K., et al. (1992) Cell 69: 989].

Melanoma-specific effector T lymphocytes have been isolated from different sources including: tumor infiltrating lymphocytes (TIL) isolated from the solid tumor [Muul, L., et al. (1987) J. Immunol. 138: 189], PBL [Vose, B.M. and Bennard, G.D. (1982) Nature 296: 359] and lymph node cells [Singcluff, C.L., et al. (1987) Arch. Surg. 122: 1407]. These cells are predominantly $CD3^+$, $CD8^+$ CTL that exhibit preferential cytolytic activity against autologous melanoma tumor cells [Muul, L., et al. (1987) J. Immunol. 138: 189 and Itoh, K., et al. (1988) J. Exp. Med. 168: 1419].

The HLA-A2.1 class I molecule, a product of the HLA-A2.1 gene, is an effective antigen-presenting molecule for presentation

of melanoma antigens to T cells [Crowley, N.J., et al. (1990) Cancer Res. 50: 492 and Kawakami, Y. (1992) J. Immunol. 148: 638]. The demonstration that HLA-A2+ melanoma-specific CTL can lyse the majority of A2+ melanomas used [Darrow, T.L., et al. (1989) J. Immunol. 142: 3329 and Hom, S.S., et al. (1991) J. Immunother. 3: 153], coupled with the finding that A2+ melanoma cells transfected with the A2.1 gene can present epitopes recognized by A2+-restricted melanoma-specific CTL [Kawakami, Y. (1992) J. Immunol. 148: 638] provide evidence for specific recognition of shared melanoma antigens by T cells. HLA-A2-derived T cell epitopes are likely to have broad utility in the development of diagnostics or therapeutics for melanoma since HLA-A2 molecules are expressed in about 50% of the Caucasian population [Tiwari, J.L. and Tetaski, P.I. (1985) In: HLA and Disease Associations, (Tiwari, J.L. and Tetaski, P.I., eds.), Springer-Verlag, N.Y., p. 10] and it is the Caucasian population in which the overwhelming instances of melanoma are found [Reintgen, D.S., et al. (1982) J.A.M.A. 248: 1856].

One approach for the direct characterization of CTL epitopes involves the reconstitution in vitro of CTL epitopes by allowing exogenously prepared peptides to sensitize target cells expressing appropriate class I molecules for specific lysis by CTL. Although this approach has been successful in the identification of some viral and alloreactive T cell epitopes [Van Bleek, G.M. and Nathenson, S.G. (1990) Nature 348: 213; Falk, K., et al. (1991) J. Exp. Med. 174: 425; Udaka, K., et al. (1992) Cell 69: 998], very few natural peptide adducts of class I MHC proteins have been isolated. This likely results, at least in part, from low levels of expression since as few as 100 complexes on the cell surface may be sufficient to stimulate T cells [Demotz, S., et al. (1990) Science 249: 1028; Falk, K., et al. (1991) J. Exp. Med. 174: 425; Christnick, E.R., et al. (1991) Nature 352: 67; Udaka, K., et al. (1992) Cell 69: 998].

660 TIL, a long-term melanoma-specific T cell line and its autologous tumor target cell line (660 mel) have been established from a human melanoma tumor sample [Reilly, E.B. and Antognetti, G. (1991) Cell. Immunol. 135: 526]. Tumor cell CTL activity of 660 TIL is inhibited by several monoclonal antibodies (MAB) including

those against CD3, CD8 and HLA-A2 molecules, indicating that the effector cells are class I-restricted $CD3^+$, $CD8^+$ CTL that recognize melanoma antigen(s) in association with HLA-A2 molecules.

Summary of the Invention

One aspect of the invention presents substantially pure tumor-associated antigens which can bind to HLA molecules on tumor and non-tumor cells and cause those cells to be recognized by and destroyed by T cells. These antigens can be obtained by the purification methods disclosed herein using different T cells and/or tumor cells. The T cells are preferably CTL and more preferably TIL. The destruction is preferably by lysis of the cells. In particular, the invention presents a substantially pure family of HLA-A2-associated tumor antigens, an example of which is T cell-specific melanoma antigens which when bound to HLA-A2 on a cell will cause the cells to be recognized and lysed by TIL. These antigens are generally between 5 to 20 amino acids in length, more preferably between 5-15, and most preferably between 7 to 12 amino acids in length. Examples of the T cell-specific melanoma antigens are peptides such as mel Ag 906 or mel Ag 1007. The molecular weight of mel Ag 906 is about 906 Dalton (D) with a $\pm$ 10% margin of error. The molecular weight of mel Ag 1007 is about 1007 Dalton (D) with a $\pm$ 10% margin of error.

Another aspect of the invention presents the polypeptide(s) from which the tumor-associated antigens are derived.

Another aspect of the invention presents methods for purifying the tumor-associated antigens, preferably from tumor cells and by means of cytotoxicity tests using T cells, in particular TIL.

Another aspect of the invention presents methods for diagnosing cancer using the above tumor-associated antigens, the polypeptide(s) from which the tumor-associated antigens are derived, or the derivatives of the above, such as their nucleotide sequences.

Another aspect of the invention presents methods for identifying cancer therapeutics using the above tumor-associated

antigens, and the polypeptide(s) from which the tumor-associated antigens are derived. The cancer therapeutics identified thereby are also presented.

Another aspect of the invention presents vaccines and methods for vaccination using the above tumor-associated antigens, and the polypeptide(s) from which the tumor-associated antigens are derived.

Description of the Drawings

Figure 1 presents the CTL activity of 660 TIL against its autologous tumor cell target, 660 mel.

Figure 2 presents tumor cell cytotoxic activity of 660 TIL after stimulation with cultured 660 mel.

Figure 3 presents the HLA-A2 affinity purification from 660 mel cells.

Figure 4 presents the cytotoxic activity of peptides derived from 660 mel HLA-A2 molecules and fractionated by reverse phase chromatography on a C18 column.

Figure 5 presents the cytotoxic activity of pooled peptide fractions 42-46 from the C18 column (see Figure 4) fractionated by reverse phase chromatography on a phenyl column.

Figure 6 presents the cytotoxic activity of pooled peptide fractions 40-41 from the phenyl column (see Figure 5) fractionated by reverse phase chromatography on a C4 column.

Figure 7 presents the cytotoxic activity of pooled peptide fractions 49-50 from the C4 column (see Figure 6) fractionated by reverse phase chromatography on a minibore C8-polymer column.

Detailed Description of the Invention

The present invention presents substantially pure tumor-associated antigens which can bind to HLA molecules on tumor and non-tumor cells and cause those cells to be recognized by and destroyed by T cells. These antigens can be obtained by the

purification methods disclosed herein using different T cells and/or tumor cells.

The T cells are preferably CTL and more preferably TIL. The destruction is preferably by lysis of the cells. In particular, the invention presents a substantially pure family of HLA-A2-associated tumor-associated antigens, an example of which is T cell-specific melanoma antigens which when bound to HLA-A2 on a cell will cause the cells to be recognized and lysed by TIL. These antigens are generally between 5 to 20 amino acids in length, more preferably between 5-15, and most preferably between 7 to 12 amino acids in length. Examples of the T cell-specific melanoma antigens are peptides such as mel Ag 906 or mel Ag 1007. The molecular weight of mel Ag 906 is about 906 Dalton (D) with a ± 10% margin of error. The molecular weight of mel Ag 1007 is about 1007 Dalton (D) with a ± 10% margin of error.

The present invention also provides a description of the isolation and characterization of such antigens. The invention further presents the polypeptide(s) from which such peptides are derived. These tumor antigens, their polypeptide(s) from which they are derived, and their derivatives may prove particularly useful as cancer diagnostics and therapeutics, including vaccines.

"Ag" is herein defined as antigen, except in the context of Ag 906 and Ag 1007 which are the names of specific peptides.

"MAB" is herein defined as monoclonal antibody.

"MHC" is herein defined as major histocompatibility complex.

"T cells" is herein defined as lymphocytes derived from the thymus. "Tumor-specific T cell" is a T cell that recognizes a specific tumor. Sources of tumor-specific T cells include but are not limited to: a tumor or tumor specimen and as such are designated TIL; primary lymph organs such as lymph nodes or spleen; and peripheral blood. Although TIL may include both CTL and T helper cells, 660 TIL is a CTL.

"TIL" is herein defined as tumor infiltrating lymphocytes

"CTL" is herein defined as cytotoxic T lymphocytes.

"Tumor-associated antigen" is hereby defined as an antigen expressed by tumor.

"T cell-specific tumor antigen" is hereby defined as a tumor-associated antigen that is recognized by a T cell. For example, "T

cell-specific melanoma antigen" denotes an antigen which is specific to melanoma and which is recognizable by a T cell.

"HLA-associated tumor antigen" is hereby defined as a tumor-associated antigen which can associate with HLA to form a complex. Preferably, this complex is recognizable by T cells with the same HLA phenotype as the tumor target cell expressing the tumor-associated antigen i.e., both cells are HLA-compatible. "HLA-A2-associated tumor antigen" is hereby defined as a tumor-associated antigen which can associate with HLA-A2 to form a complex. Preferably, this complex is recognizable by HLA-A2+ T cells. These T cells may include CTL and are preferably derived from tumors, peripheral blood, spleens or primary lymph organs. The most preferred T cell is TIL. The most preferred TIL is 660 TIL.

Specific Ag recognition by T cells is based on binding of the T cell receptor to a complex composed of an antigenic peptide and an MHC molecule. There are two main varieties of MHC antigens, namely class I and class II. There are dozens of distinct class I and class II antigens. Class I antigens (of which the HLA-A2 molecule is one) are present on almost all cells and it is the unique combination of class I MHC antigens that mark those cells of each individual as unique. Most cells express between two to four distinct class I antigens. Although there are dozens of class I MHC antigens, HLA-A2 is probably the most prevalent, present in about 50% of the Caucasian population. This means that, although these individuals still have distinct haplotypes (i.e. the unique display of MHC antigens on the surface of their cells), there may be some common sharing. Since class I MHC antigens are also responsible for binding small peptides to serve as T cell antigens, this also means that all individuals who express HLA-A2 are likely to express many of the same T cell antigens. HLA-A2-restricted melanoma-specific CTL (also referred to as A2-restricted melanoma-specific CTL) are, therefore, T cells that recognize the combination of the HLA-A2 antigen associated with a small peptide.

The HLA-A2 molecule is a product of the HLA-A2 gene. Humans who express the A2 protein are classified as HLA-A2+. HLA-A2 class I molecule is also referred to as the A-2 protein.

There are many subtypes of HLA-A2 molecules (e.g. A-2.1, A-2.2, A-2.3, etc.) [Parnham, P., et al. (1991) Immunogen. 33: 310], each of which is coded for by a different gene, although the HLA 2.1 subtype is the most prevalent.

Mature peripheral T cells with an αβ (the most common) T cell receptor can be categorized into two major classes expressing either the CD4 or CD8 transmembrane glycoprotein. $CD4^+$ T cells (which generally include T helper cells) recognize peptide fragments bound to MHC class II molecules, while $CD8^+$ T cells (which generally include CTL) recognize peptide fragments bound to MHC class I molecules.

CTL epitopes are complexes composed of antigenic peptides and class I MHC molecules. Antigenic peptides arise by cleavage of endogenously synthesized proteins that are bound by newly synthesized class I molecules in the endoplasmic reticulum and subsequently transported to the cell surface [Tsomides, T.J. and Eisen, H.N. (1991) J. Biol. Chem. 266: 3557]. Thus, a general scheme for isolation of such peptides consists of expansion of cultures of a melanoma cell line known to express the proper epitopes. The use of this approach to identify medically relevant melanoma antigens could lead to determination of the amino acid sequences of these peptides and identification of the proteins from which they are derived. This information could provide a basis for diagnostic and therapeutic intervention.

Methods for Obtaining Tumor-Associated Antigens

There are several methods for obtaining the T cell-specific tumor antigens; disclosed herein are Methods 1 and 2. Method 1 involves purifying antigens from the membrane of tumor cells. Preferably, the antigens are selected for their binding to HLA. Such selection can be achieved using HLA affinity chromatography. The resulting peptides are then purified by HPLC fractionation. Peptides from each HPLC fraction are then mixed with an HLA-compatible non-tumor target cell and selected for their ability to induce lysis of those target cells by T cells. That is, the T cell recognizes the complex of HLA and the tumor-associated antigen,

and thereby bind to the non-tumor cell and causes its lysis. The tumor cell, non-tumor cell and T cell have to be HLA-compatible. The tumor cell and T cell can be autologous. The non-tumor cell can be non-autologous.

Method 2 does not rely on HLA affinity columns, therefore, the non-tumor cell (target cell) need not be HLA-compatible with the tumor cell; it needs only be HLA-compatible with the T cell (effector cell) in the test for cytotoxicity. The tumor, non-tumor, and T cells need not be autologous.

In both of the above methods, the T cell is preferably derived from tumor, lymph, spleen, or peripheral blood. More preferably, the T cell is a CTL. In the methods presented herein, preferably, a T cell which is specific to a particular tumor (i.e. a tumor-specific T cell) is used to detect the tumor-associated antigen derived from the specific tumor. For example, in the preferred embodiment, the tumor is melanoma and the tumor-specific T cell is a melanoma-specific T cell, i.e. a T cell which recognizes a melanoma tumor cell. Upon recognition of its target tumor cell, the tumor-specific T cell is capable of lysing that target cell or releasing lymphokines or cytokines. Tumor cell lines and T cells which are specific to them are known in the art and can be obtained by methods known in the art (see e.g. Reilly, E.B. and Antognetti, G. (1991) Cell. Immunol. 135: 526 and Topalian, S.L., et al. (1987) J. Immunol. Methods 102: 127). For example, in the most preferred embodiment, the T cell is a melanoma-specific T cell line 660 TIL; and the tumor cell is a melanoma cell line 660 mel. Examples of other tumor cells include those from colon, ovarian, cervical, lung, breast, prostate, and renal tumors.

It is to be noted that different T cells may preferentially recognize different tumor-associated antigens in the pool of HPLC fractions from the same tumor cell. Similarly, different tumor cells may express different tumor-associated antigens that are recognized by same or different T cells. The tumor-associated antigen which would be of wide use, would be the antigen which is widely expressed on the tumor cells of different individuals. This tumor-associated antigen can be either recognized by a specific T cell of an individual or T cells of different individuals.

The following describes Method 1.

Method 1

Since T cell epitopes may be expressed at levels as low as 100 molecules per cell, the cell expansion required may be substantial, leading to the generation of more than 10^{11} cells. Detergent solubilized membranes prepared from these cells may then be passed over antibody affinity columns to isolate class I MHC molecules to which the tumor-associated antigens are complexed (herein called the "MHC-tumor-associated antigen complexes"). This can be achieved using methods known in the art.

The MHC-tumor-associated antigen complexes are then denatured to release the tumor-associated antigens. This can be achieved using acidic treatment with methods known in the art, such as incubation with trifluoroacetic acid. The released tumor-associated antigens are then collected by ultrafiltration [as described in Udaka, K., et al. (1992) Cell 69: 989] and subjected to HPLC fractionation. This is required since the mixture of tumor-associated antigen released from MHC molecules is very complex. Tumor-associated antigen fractions are tested for biological activity on the basis of ability to sensitize MHC-compatible non-tumor target cells for susceptibility to lysis by T cells. For example, in the case where the tumor-associated antigens are derived from melanoma tumor cells, the fractions can be tested for biological activity on the basis of ability to sensitize MHC-compatible non-tumor target cells for susceptibility to lysis by melanoma-specific CTL, preferably by TIL, and more preferably by 660 TIL. In this manner active fractions can be pooled and re-fractionated on HPLC under different sets of conditions for additional purification. Successive reverse phase columns such as HPLC are the preferred purification methods because, unlike ion exchange columns and gel filtrations, the antigens are not in salt or non-volatile buffers. Thus, with reverse phase columns, no desalting step is required and the fractions can be directly tested for cytotoxic activity, thus avoiding further loss of antigens, which is crucial in view of the potentially miniscule amounts of these antigens expressed by the cells. Examples of reverse phase

columns are those using alkyl sidechains (e.g. C1, C2, C4, and C18), and cyano sidechains. In the Example below, the C18, phenyl, C4, C8-polymeric fractionations can be employed in any order.

Following several rounds of HPLC fractionation the quantity, purity and molecular weight of the tumor-associated antigens in the active HPLC fractions can be assessed by physical means such as mass spectroscopy. The quantity of material present will determine the approach to be used for amino acid sequence analysis. Sequencing by Edman degradation generally requires >10 pmol of material. Therefore, the isolation of less material will necessitate the use of tandem mass spectrometry to generate sequence information.

Method 2

Method 2 involves an acid extraction of low molecular weight materials from cells. The tumor cells are acid extracted, and peptides of less than 10,000 daltons are recovered by ultrafiltration methods known in the art. For example, the tumor cells can be extracted in TFA, centrifuged at 31,000 x g for 30 min and the supernatant subjected to ultrafiltration through a Centricon 10 membrane (Amicon, Lexington, MA) [Udaka, K., et al. (1992) Cell 69:998]. Filtrates are dried by SpeedVac and redissolved in 0.1% TFA for reverse phase HPLC.

Determination of T Cell Specificity for Tumor-Associated Antigens

The purified tumor-associated antigen can be determined to be T cell-specific tumor antigen by several means. The first means is disclosed under Method 1, wherein the determinant is the ability of the tumor-associated antigen to render non-tumor cell susceptible to lysis by T cells. These T cells are preferably CTL and TIL.

Alternatively, the determinant can be the ability of the tumor-associated antigen to cause non-tumor cell to trigger the release of cytokines or lymphokines by T cells. These T cells are preferably CTL and helper T cells. The cytokines or lymphokines that can be tested include: interferons, interleukins, and granulocyte macrophage colony stimulating factors. The methods

for determining the release of cytokines are known in the art, such as enzyme-linked immunoabsorbent assay (ELISA) [Schwartzentruber, D.J., et al. (1991) J. Immunol. 146: 3674] and polymerase chain reaction (PCR) [Carding, S.R., et al. (1992) J. Immunol. Methods 15: 277].

Sequence Determination

Upon sequence determination of the tumor-associated antigens, such as (an) active peptide(s), it will be possible to synthesize the peptide(s) chemically. The peptide(s) will be purified using methods known in the art, for example, by C18 reverse phase HPLC (Vydac, Hesperia, CA) and biological activity will be confirmed by the ability to reconstitute epitopes for the T cell used as effector cells, e.g. in the Example below, the effector cell is HLA-A2-restricted 660 TIL.

Class I HLA-associated tumor Ags purified by the method disclosed herein are preferably recognized by class-I HLA-restricted T cells. Preferably the Ags are expressed in HLA-A2+ tumor cells or are associated with HLA-A2+ molecules on the surface of tumor cells. These Ags may be recognized by T cells derived from tumors (TIL), peripheral blood or primary lymph organs such as lymph nodes and spleen. Thus, for the purpose of screening for or identifying the HLA-A2-associated tumor antigen, members of the above T cells, besides 660 TIL, may be used. Methods for growing, harvesting and maintaining T cells in general, and HLA-A2+ T cells in particular, are known in the art and are described [e.g., Topalian, S.L., et al. (1987) J. Immunol. Methods 102: 127 and Reilly, E.B., et. al. (1990) J. Immunol. Methods 126: 273].

Once the tumor-associated antigen has been sequenced, it can also be cloned into a host cell and produced recombinantly.

The tumor-associated antigen isolated and purified as disclosed in this patent application may have been derived by any of the following means:

(1) the gene for a full length protein (an example of a "starting polypeptide") could be overexpressed in the tumor cell compared to lower levels or absence of expression in normal cells;

(2) the gene for a full length protein (another example of "starting polypeptide") expressed in an altered form may result in expression in the tumor cell of an altered form of a full length protein compared to a normal cell;

(3) a full length protein (as described in (1) and (2) above) could be expressed in the tumor cell and processed to generate a polypeptide of a different length expressed either intracellularly or on the cell surface in association with HLA molecules (the polypeptide is herein termed "end-product polypeptide");

(4) a full length protein (as described in (1) and (2) above) could be expressed in the tumor cell and processed to produce various peptides which are present in the cell or expressed on the cell surface in association with HLA molecules (these polypeptide(s) are also termed "end-product polypeptide(s)").

Different polypeptides can be expressed with different HLA molecules. These polypeptides may arise by differential processing of the same full length protein or, in fact, processing of different full length proteins. That is, a specific polypeptide may be expressed by a HLA-A2+ individual which would complex with the HLA-A2 protein and be recognized by a T cell, whereas another polypeptide may be expressed by the same or a different gene in an individual expressing another HLA protein (such as HLA-B7) which would complex with that HLA protein and be recognized by a T cell. The T cells in both cases may not be the same; nor the tumor cells from which the polypeptides are derived. Thus, another aspect of this invention presents other T cell-specific tumor antigens which can associate with HLA molecules, and which can be obtained by the purification methods disclosed herein using different T cells and/or tumor cells.

Thus, encompassed within this invention and the term "polypeptide", would be the tumor-associated antigen, the differently processed or expressed polypeptides, and the full length polypeptides (whether starting or end-product polypeptides) which may be the origins of the tumor-associated antigen purified by the method disclosed herein and which may be found in the pool of polypeptides purified by this means. That is, within the pool of the purified polypeptides, there may be polypeptides that are at different stages of the processing or modification phases. Thus, the T cell-specific melanoma antigens such as mel Ag 906 and mel Ag 1007, can be fragments of larger polypeptides or the processed products of starting polypeptides or groups of polypeptides, or fragments of end-product polypeptides. Thus, besides the end-product polypeptides, or fragments of polypeptides, the starting polypeptides would also be included within this invention. More preferably, the starting and end-product polypeptides share at least 78-100%, and more preferably at least 87-100%, and most preferably, 100% homology in amino acid residues with the tumor-associated antigen purified by the method disclosed herein for the segment in which the starting (or end-product) polypeptide and the tumor-associated antigen are most closely aligned.
The preferred example of this polypeptide is Class 1 HLA-associated tumor antigen, and more preferably an HLA-A2-associated tumor antigen and its starting and end-product polypeptides.

Characterization of a tumor-associated antigen should make it possible to determine the mature protein from which it is derived in one of two ways. The identity of the parent protein may be deduced on the basis of sequence homology searches. If necessary, the gene for the protein of interest could be determined by screening DNA libraries, prepared from the tumor cells from which the tumor-associated antigen originated, with oligonucleotide probes corresponding to the peptide sequence.

<u>HLA-A2-Associated Epitopes are Shared by Different Melanoma Lines</u>

In the case of the 660 mel, the active peptide derived from 660 mel will be reconstituted with HLA-A2 to determine if this

CTL epitope can be recognized by other A2-restricted melanoma-specific CTL. This will provide a better understanding for the existence of shared melanoma antigens.

Although previous studies suggest the existence of shared melanoma antigens recognized by CTL, it is likely that multiple naturally occurring shared peptide-defined CTL epitopes may be presented by melanoma cells. Reconstitution experiments with 660 mel peptides may identify epitopes recognized by other HLA-A2-restricted melanoma-specific CTL. Determination of the amino acid sequences from the peptides and identification of the protein(s) from which they are derived will be important for the development of melanoma-specific diagnostics and therapeutics.

DIAGNOSTICS

The polypeptides obtained by the methods disclosed in this patent application, such as those purified from tumor cells, can be used to diagnose cancer, serve as cancer therapeutics or vaccines for the cancers from which the tumor cells are derived from. These polypeptides may also be expressed by tumor cells besides those that they are derived from; thus, the cancers to be detected, treated, or vaccinated against can be those other than the original tumor cells.

In the preferred embodiment, HLA-A2 derived CTL epitopes are especially likely to have broad utility in the development of diagnostics or therapeutics for melanoma because of the frequency of HLA-A2 expression. Several possible modes of action for diagnostic intervention are envisioned based on host reactivity to these antigens. For example, candidates at risk for melanoma could be screened for the presence of specific antigens by either immunoassays or DNA probes. PCR-based assays may be also prove useful for sensitive detection of antigens. Additionally, a skin test to asses melanoma-specific CTL activity may prove useful as a diagnostic tool. It is also possible that an in vitro correlation of the skin test, whereby the effector function of melanoma-specific CTL may be determined from a source such as peripheral blood, could prove valuable as well as non-invasive.

Thus, upon identification of a T cell-specific antigen recognized by melanoma-specific T cells and the full length

protein from which it is derived, it may be possible to develop diagnostic tests for their presence which may be useful in cancer detection in individuals at risk for melanoma.

1. Diagnostic tests based on detection of Ag.

a. MAB

MAB directed against the T cell-specific tumor antigen such as a peptide, or full length protein from which it is derived, will be generated by methods familiar to those who are skilled in the art. Skin specimens, obtained upon biopsy, will be used as test substrates for antibody reactivity. Although it is unlikely that a peptide-specific MAB will be able to react with a T cell Ag buried within the cleft of the HLA molecule on the cell surface, other methods of antibody reactivity will also be analyzed to detect intracellular antigens. These will include both immunofluorescence and ELISA on cell extracts, histological samples, lysed cells, biological fluid such as blood, serum, urine, breast secretion, mucous of lungs, cervical swab, cerebral fluid, and synovial fluid. These samples can be taken, for example, for detecting colon, ovarian, lung, breast, cervical, prostate and renal tumors. Both immunofluorescence and ELISA procedures are well known to those who are skilled in the art. Polyclonal and monoclonal antibodies directed against these T cell-specific tumor antigens can be made using methods known in the art. For example, antibodies can be produced by injecting a host animal such as rabbit, rat, goat, mouse etc. with the tumor-associated antigen and fragments thereof, alone or conjugated to an appropriate carrier if required to elicit an antibody response. It will be appreciated by those skilled in the art that the MAB can be produced, for example, by means of the hybridoma techniques.

b. Nucleic acid-based assays

Skin specimens obtained by biopsy may be used as source of RNA prepared by conventional methods. A chemically tagged or radiolabeled oligonucleotide encoding the T cell-specific tumor antigen can be used as a hybridization probe to detect the presence or prevalence of the messenger RNA (mRNA) of the T cell-specific tumor antigen. Where the tumor is caused by an altered gene, hybridization, PCR or ligase chain reaction (LCR) can be used to detect the altered gene or its mRNA from its wild type counterparts. Such detection methods are known in the art. The samples that can be tested include biological fluid, tissue or cells.

In similar fashion, oligonucleotides specific for the T cell-specific tumor antigen can be used to prime the PCR from template RNA isolated from biopsied samples by standard methods known in the art.

2. Diagnostic tests based on T cell reactivity

a. <u>In vivo</u> analysis

An <u>in vivo</u> skin test may provide a quick and easy diagnostic test for detection of melanoma-specific T cells and thereby serve as a useful prognosticator of melanoma in patients. Synthetic peptide(s) corresponding to the T cell-specific tumor antigen will be solubilized in balanced salts and injected intradermally. The site of the injection will be monitored for several days for evidence of a delayed type hypersensitivity (DTH) reaction which would be indicative of specific T cell immunoreactivity.

The ability to use skin tests to monitor specific cytotoxic T cell activity has been demonstrated in animal models [Kundig, T.M., et al. (1992) Proc. Natl. Acad. Sci. USA. 89: 775].

b. In vitro analysis

T cells enriched from peripheral blood samples of patients to be screened will be placed in short term culture with a HLA-A2 melanoma cell line expressing the T cell-specific tumor antigen. Increases in specific cytokine production, as measured by either ELISA or PCR, will be used to indicate specific T cell immunoreactivity. Alternatively, specific T cell immunoreactivity may be measured against HLA-A2 target cells sensitized in culture with the T cell-specific melanoma antigen. Procedures for setting up short-term cultures and using ELISA or PCR-based methods to screen for immunoreactivity are known to those skilled in the art.

THERAPEUTICS

With respect to therapeutic intervention, identification of melanoma-specific antigens may provide the basis for targeted anticancer therapy. Immunization of HLA-A2+ melanoma patients with melanoma antigens may be an effective scheme for stimulating melanoma-specific CTL and reducing or eradicating tumor cells.

T cell-specific melanoma antigen identified by the methods outlined herein may be the basis of useful immunotherapeutic strategies such as the following.

1. Patients with melanoma

Melanoma patients who produce the T cell-specific tumor antigen(s) described in this application may be immunized with the specific antigen for the purpose of stimulating a potent T cell response.

Optimal routes of administration, dose and formulation to effect T function will be explored. The progression or improvement of the diseased state will be monitored by means known in the art and may include monitoring tumor shrinkage or measuring enhancement of T cell-reactivity.

2. Vaccine

T cell-specific melanoma antigens may be used as vaccines to protect individuals at risk for melanoma. In this approach T cell-specific tumor antigens would be administered to induce the expansion of protective T cells. Optimal routes of administration, dose and formulation to effect specific CTL function would be explored.

The above therapeutics can be similarly applied to other tumor-associated antigens obtained by the methods disclosed herein for treatments of the same or different tumors. The invention also covers such pharmacological compositions such as the vaccines and therapeutic compositions disclosed herein.

EXAMPLE

Example 1

1. Materials and Methods

Cell Lines:

660 mel and 660 TIL which are autologous (derived from the same individual), 663 mel [Reilly, E.B. and Antognetti, G. (1991) Cell. Immunol. 135: 526], and M21 [Gillies, S.D. and Wesolowski, J.S. (1990) Hum. Antibod. Hybridomas 1: 47] were used. 660 TIL are TIL that recognize 660 mel, but more specifically they recognize the combination of a peptide associated with HLA-A2 antigens on the surface of the 660 mel.

Expansion of 660 mel. 660 mel were grown in 850 cm^2 roller bottles until confluent. Cells were recovered by treatment with 5 mM EDTA (pH 8.0), washed once with phosphate-buffered saline (PBS) and stored frozen at -70°C.

Membrane Extraction:
Cells were lysed at 4°C in hypotonic medium [10 mM Tris-HCl (pH 8.1), 1mM $MgCl_2$, 1mM KCl] in the presence of 100 μM phenylmethanesulfonyl fluoride, 200 μM dithiothreitol and 0.02% NaN_3 [Springer, T.A. et al. (1977) J. Biol. Chem. 252: 4682]. Membranes were collected by centrifugation for 60 min at 105,000 x g and solubilized in 3% Brij 58 (Brij 58 is available from Pierce Chemical Company, Rockford, IL) [Tsomides, T.J., et al. (1991) Proc. Natl. Acad. Sci. USA 88: 11276].

HLA Purification:
Membranes were passed over four affinity columns arranged in series as follows: Sepharose only, mouse pooled immunoglobulin-Sepharose (to bind Fc receptors and other membrane proteins) and two successive PA2.1-Sepharose (anti-HLA-A2) (Monoclonal antibody PA2.1 coupled to Sepharose resin; as described in Parham, P. (1979) J. Biol. Chem. 254: 8709). The affinity columns were prepared by Staphylococcal protein A purification of the MAB, periodate oxidation of the carbohydrate moiety in the Fc domains, and covalent coupling of the MAB to hydrazide-modified Sepharose (Pierce Chemicals, Rockford, IL.). After washing with 30 column volumes of buffers, each column was eluted with 50 mM diethylamine-HCl (pH 11) into 2 ml fractions and neutralized with 1M Tris-HCl (pH 8).

HLA-Bound Peptide Elution:
Purified HLA-A2 was concentrated to remove unbound peptides by ultrafiltration at 5000 x g (Centricon C10) and was then denatured with 1.0% trifluoroacetic acid [Rotzschke, O., et al. (1990) Science 249: 283] for 60 min at 37°C. The released peptides were collected by ultrafiltration at 5000 x g and subjected to HPLC fractionation.

HPLC Fractionation of HLA-Associated Peptides:
HPLC fractionations were performed on reverse phase columns (C18, phenyl, C4, C8-polymeric; commercially available from Vydac, Hesperia, CA) using a binary gradient system and detection by ultraviolet absorbance at 220 nm. The first three chromatographies on C18, phenyl, and C4 silica-based columns (4.6 x 250 mm) were carried out with solvent A = 0.1% trifluoroacetic acid (TFA) in H_2O, solvent B = 0.085% TFA in acetonitrile, a gradient of between 0.25 and 1% B per minute, and a flow rate of 1 ml/min. For the fourth and final chromatography using a C8-polymer column (2 x 250 mm), solvent A was 125 mM triethylamine acetate (TEAAc) in H_2O (pH 7.1), solvent B was 75 mM TEAAc in acetonitrile, and the flow rate was 0.25 ml/min. Following each round of chromatography, fractions collected at 0.5 or 1 min intervals were dried by SpeedVac (Savant, Westbury, NY) and resolubilized for cytotoxicity assays.

Cytotoxicity Assay:
To measure lysis due to melanoma-specific peptide 2.5 x 10^3 ^{51}Cr-labeled JY target cells in 50 µl of RPMI media with 10% heat-inactivated fetal bovine serum were incubated with 50 µl of peptide-containing samples prepared in RPMI for 1-3h at 37°C. 660 TIL in RPMI with 10% heat-inactivated fetal bovine serum were then added at a E:T ratio of 50:1 in a final volume of 200 µl. The cells were incubated at 37°C for 4 h and

the amount of ^{51}Cr released into the extracellular medium was determined. The percent specific lysis was calculated as 100 x (^{51}Cr released - spontaneous release)/(total release in HCl - spontaneous release). Toxicity controls consisted of peptide plus ^{51}Cr-labeled target cells without addition of 660 TIL.

Mass Spectrometry:
The active fractions from the final chromatography were analyzed by mass spectrometry to determine the molecular weight.

2. Results

660 TIL Cytotoxic Activity Is Directed towards 660 mel:
660 TIL were tested for lytic activity against both autologous and non-autologous melanoma tumor cell lines. Figure 1 presents the CTL activity of 660 TIL against its autologous tumor cell target, 660 mel. Autologous (660 mel) and non-autologous (663 mel and M21) human melanoma cell lines were radiolabeled with ^{51}Cr and incubated in 4 h assays with 660 TIL at an effector:target (E:T) ratio of 50:1. Supernatants were collected, the radioactivity determined and used as an indicator of the lytic activity of 660 TIL as measured by % ^{51}Cr release. As shown in Figure 1, significant levels of cytotoxic activity were apparent only when the autologous tumor cell line 660 mel was used as a target cell.

Stimulation of 660 TIL Cultures with 660 mel Increases Specific Cytotoxic Activity:
660 TIL were maintained in culture for several months and then stimulated repeatedly over the course of several weeks with the addition of 660 mel cells to the culture at a T cell/target cell ratio of 10:1. Figure 2 presents tumor cell cytotoxic activity of 660 TIL after

stimulation with cultured 660 mel. Lytic activity of TIL stimulated once (open bars) or three times (hatched bars) was determined in 4 h ^{51}Cr release assays at E:T ratios shown. Error bars represent standard deviation.

Figure 2 shows that multiple rounds of tumor cell stimulation over the course of several weeks increased the specific 660 TIL cytotoxic activity to greater than 70% lysis at an E:T ratio of 50:1.

660 TIL are $CD3^+$, $CD8^+$ CTL that Recognize Ag Associated with Class I MHC Molecules:

The effect of several MAB on the lysis of autologous melanoma target cells by 660 TIL was determined as shown in Table I. Table I presents the effect of MAB on the lysis of 660 mel cells by 660 TIL at different E:T ratios. MAB specific for CD3 (OKT3 is available from the American Type Culture Collection, Rockville, MD , herein referred to as ATCC. OKT3's ATCC Accession No. is CRL 8001), CD4 (OKT4, ATCC Accession No. CRL 8002), class I MHC molecules (PA2.6, ATCC Accession No. HB118) and cellular adhesion molecules (anti-ICAM and anti-LFA-1. Both are commercially available from AMAC, Inc., Westbrook ME.) were added to radiolabeled 660 mel cells at a final concentration of 50 μg/ml and incubated for 30 min at room temperature. 660 TIL were then added, incubation was continued for 4 h at 37°C and the assay was harvested.

TABLE 1

EFFECT OF MAB ON LYSIS OF TUMOR CELLS BY AUTOLOGOUS TIL

MAB[a]	% LYSIS[b] E:T RATIO	
	25:1	5:1
None	46 ± 0.3[c]	21 ± 3.8
OKT3	3 ± 0.2	2 ± 0.3
OKT8[d]	35 ± 2.3	9 ± 1.4
PA 2.6	7 ± 1.4	2 ± 0.2
Anti-ICAM	28 ± 3.1	9 ± 0.3
Anti-LFA-1	11 ± 1.4	3 ± 0.2

a MAB used at a final concentration of 50 μg/mL

b Results of a 4 h ^{51}Cr release assay

c Standard deviation calculated from duplicates

d Inhibition by OKT8 is enhanced in the presence of higher concentrations of the MAB.

In agreement with classical CTL function, 660 mel lysis by 660 TIL was inhibited by MAB to CD3, CD8 and class I MHC molecules. MAB against the intercellular adhesion molecules ICAM and LFA-1 also inhibited lysis of tumor cells.

660 TIL Recognize Ag Associated with HLA-A2 Molecules:

Table 2 presents the effect of anti-HLA-A2 MAB on the lysis of 660 mel cells by 660 TIL at different E:T ratios. MAB specific for HLA-A2 (BB7.2, ATCC Accession No. HB82) as well a control MAB specific for another HLA molecule, HLA-B7 (BB7.1, ATCC Accession No. HB56) were added to radiolabeled 660 mel cells at a final concentration of 50 μg/ml and incubated for 30 min at room temperature. 660 TIL were then added, incubation was continued for 4 h at 37^{o}C and the assay was harvested.

TABLE 2

EFFECTS OF ANTI - HLA CLASS I MAB ON LYSIS OF 660-MEL BY AUTOLOGOUS TIL

MAB[a]	% LYSIS[b] E:T RATIO 50:1	10:1
None	48 ± 0.4[c]	34 ± 0.3
BB7.2	4 ± 0.5	10 ± 3.9
BB7.1	55 ± 0.5	38 ± 1.1

a MAB used at a final concentration of 50 μg/mL

b Results of a 4 h ^{51}Cr release assay

c Standard deviation calculated from duplicates

Table 2 shows that the recognition of 660 mel by 660 TIL was inhibited by MAB against HLA-A2 (BB7.2), but not by MAB against directed another MHC molecule, HLA-B7 (BB7.1). The recognition of Ag on the established melanoma cell line is, therefore, restricted by HLA-A2.

660 Mel Cells Can Be Expanded to Large Numbers for Extraction of Cell Membranes:

5×10^{11} 660 mel cells were grown as monolayers in more than 4000 850 cm^2 roller bottles and recovered by treatment with 5 mM EDTA (pH 8.0). After washing cells with PBS and storage of the cell pellets at -70°C, membranes were extracted from batches of 5×10^{10} cells by lysis in hypotonic medium [10 mM Tris-HCl (pH 8.1), 1 mM $MgCl_2$, 1 mM KCl] in the presence of 100 μM phenylmethanesulfonyl fluoride, 200 μM dithiothreitol and 0.02% NaN_3 at about 2.5×10^8 cells/ml hypotonic media. Extracted cells were centrifuged at 1000 x g for 10 min at 4°C . The supernatants were collected and the cell pellet was re-extracted. A minimum of eight successive extractions ranging in time from 1-12 h were performed on each batch of cells. Membranes were collected from pooled supernatants by centrifugation for 60 min at 105,000 x g, and solubilized in 3% Brij 58 in hypotonic lysis buffer.

HLA-A2 Affinity Purification:

Solubilized membranes in batches derived from about 5×10^{10} cells each were passed over four 5 ml affinity columns arranged in series as follows: Sepharose only, mouse pooled immunoglobulin-Sepharose (to bind Fc receptors and other membrane proteins) and two successive PA 2.1-Sepharose (anti-HLA-A2). The affinity columns were prepared by Staphylococcal protein A purification of the MAB, periodate oxidation of the carbohydrate moiety in the Fc domains and covalent coupling of about 3-4 mg of the MAB per ml of hydrazide-modified Sepharose. Final coupling yields were generally in the range of 1.5-2.5 mg MAB/ml Sepharose. Columns were first washed extensively with ten column volumes of 0.1 M Tris-HCl (pH 8.0), 0.2% Brij 58, 10 μM phenylmethanesulfonyl fluoride ("wash buffer"). After passage of the membranes, the columns were separated and washed with ten column

volumes of each of the following: 1) wash buffer; 2) 1M Tris-HCl (pH 8.0), 0.2% Brij 58; and 3) 0.02 M Tris-HCl (pH 8.0). Bound material was eluted from the columns with 50 mM diethylamine-HCl (pH 11) and 2 ml fractions were collected into tubes containing 0.2 ml 1M Tris-HCl (pH 8.0). Figure 3 presents the HLA-A2 affinity purification from 660 mel cells. Membranes extracted from 660 mel cells were passed through affinity columns prepared with MAB PA2.1, ATCC HB117 (anti-HLA-A2) and after extensive washing of the column specifically bound HLA-A2 molecules were eluted in the presence of 50 mM diethylamine-HCl (pH 11).

Figure 3 shows the results of a typical elution profile from the mouse immunoglobulin and the two successive PA2.1 columns. Figure 3 presents the HLA-A2 affinity purification from 660 mel cells. HLA purity was assessed by SDS/PAGE. Yields and purity were further determined by quantitative amino acid analysis.

HLA-A2-Bound Peptide Elution:
Purified HLA-A2 was purified and concentrated to remove unbound peptides by ultrafiltration at 5000 x g (Centricon C10) and then denatured with 1.0% trifluoroacetic acid for 60 min at 37°C. The released peptides were collected by ultrafiltration at 5000 x g and subjected to HPLC fractionation.

Purification of Antigen Reactivity Associated with HLA-A2:
HPLC fractionations were performed on reverse phase columns C18, phenyl, C4, C8-polymeric using a binary gradient system and detection by ultraviolet absorbance at 220 nm. The first three chromatographies on C18, phenyl, and C4 silica-based columns (4.6 x 250 mm) were carried out with solvent A = 0.1% trifluoracetic acid (TFA) in H_2O, solvent B =

0.085% TFA in acetonitrile, a gradient of between 0.25 and 1% B per min, and a flow rate of 1 ml/min. For the fourth and final chromatography using a C8-polymer column (2 x 250 mm), solvent A was 125 mM triethylamine acetate (TEAAc) in H_2O (pH 7.1), solvent B was 75 mM TEAAc in acetonitrile, and the flow rate was 0.25 ml/min. Following each round of chromatography, fractions collected at 0.5 or 1 min intervals were dried by SpeedVac and resolubilzed for cytotoxicity assay.

Cytotoxic Assays to Evaluate Antigen Purity:
Peptide-containing HPLC fractions in 50 µl of RPMI were added to 50 µl ^{51}Cr-labeled J-Y target cells (2.5 x 10^3 cells) in RPMI plus 10% heat inactivated fetal bovine serum and incubated at 37°C for 1 to 3 hours. One-hundred µl of 660 TIL in RPMI plus 10% heat-inactivated fetal bovine serum were then added to an E:T ratio of 50:1 and the cells were incubated at 37°C for 4 h. The amount of ^{51}Cr in the extracellular medium was determined and specific lysis was calculated.

Figure 4 presents the cytotoxic activity of peptides derived from 660 mel HLA-A2 molecules. Peptides released from HLA-A2 molecules by treatment with trifluoroacetic acid were fractionated by reverse phase chromatography on a C18 column. Fractions were collected at 1 min intervals at a flow rate of 1 ml/min and subjected to standard cytotoxicity assays using ^{51}Cr-labeled JY cells (an HLA-A2$^+$ non-tumor EBV-transformed human B cell line). The two major peaks of cytotoxic activity corresponded to fractions 42-46.

Figure 5 presents the cytotoxic activity of pooled peptide fractions 42-46 from the C18 column (see Figure 4) fractionated by reverse phase chromatography on a phenyl column. Fractions were collected at 1 min intervals at a flow rate of 1 ml/min and subjected to standard cytotoxicity assays using ^{51}Cr-labeled JY cells. The major peak of cytotoxic activity corresponded to fractions 40-41.

Figure 6 presents the cytotoxic activity of pooled peptide fractions 40-41 from the phenyl column (see Figure 5) fractionated by reverse phase chromatography on a C4 column. Fractions were collected at 1 min intervals at a flow rate of 1 ml/min and subjected to standard cytotoxicity assays using ^{51}Cr-labeled JY cells. The major peak of cytotoxic activity corresponded to fractions 49-50.

Figure 7 presents the cytotoxic activity of pooled peptide fractions 49-50 from the C4 column (see Figure 6) fractionated by reverse phase chromatography on a minibore C8-polymer column. Fractions were collected at 0.5 min intervals at a flow rate of 0.25 ml/min and subjected to standard cytotoxicity assays using ^{51}Cr-labeled J-Y cells. Active fractions correspond to 83-85.

Mass Spectrometry to Characterize Active Peptide:
The active fractions from the final chromatography (as represented by fractions 83-85 in Figure 7) were analyzed by mass spectrometry to determine purity and molecular weight similar to the method disclosed in Hunt, D. F., et al. (1992) Science 255: 1261. There were several peptides in the fractions. The two most prevalent peptides, designated mel Ag 906 and mel Ag 1007 were identified. The molecular weight of mel Ag 906 is about 906 Dalton (D) with a ± 10% margin of error. The molecular weight of mel Ag 1007 is about 1007 Dalton (D) with a ± 10% margin of error. The amino acid sequence can be determined by similar tandem mass spectrometry.

Peptide Synthesis of Active Peptide:
Upon sequence determination of (an) active peptide(s) it will be possible to synthesize that peptide chemically. The peptide(s) will be purified by C18 reverse phase HPLC and biological activity will be confirmed by the ability to reconstitute epitopes for the HLA-A2-restricted 660 TIL.

Identifying the Precursor, End-Product, or Related Polypeptides:

Once the partial or full sequence of an active polypeptide has been determined, the sequence is matched by homology using a program such as String Search with the University of Wisconsin GCG sequence analysis software package to determine the precursor, end-product or related polypeptides.

Alternatively, the partial DNA sequence of the polypeptide can be used as hybridization probes to identify the gene which encodes the polypeptide from a cDNA library prepared from the tumor cell line from which the polypeptide has been purified from or any tumor cell line. Thus, the polypeptide can also be identified. This can be achieved using methods known in the art.

HLA-A2-Associated Epitopes are Shared by Different Melanoma Lines:

The active peptide derived from 660 mel will be reconstituted with HLA-A2 to determine if this CTL epitope can be recognized by other A2-restricted melanoma-specific CTL. This will provide a better understanding for the existence of shared melanoma antigens.

Discussion/Conclusion

Results indicate that 660 TIL, derived from a solid tumor sample of a patient with malignant melanoma, is a $CD3^+$, $CD8^+$ CTL that has specific lytic activity directed towards its autologous tumor cell line, 660 mel. The peptide recognized by 660 TIL is a short peptide of about 7-10 amino acids in length associated with HLA-A2 molecules expressed on the surface of 660 mel. 660 mel cells are amenable to large scale expansion in tissue culture while still retaining their properties as antigen-specific-target cells.

Cells prepared in this manner have been used as a source of membranes for the subsequent affinity purification of complexes of HLA-A2 molecules and their associated peptides. The dissociation of endogenous peptides from HLA-A2 molecules results in a complex mixture of peptides all of which are expected to compete for binding to the HLA-A2 molecules. Multiple rounds of HPLC fractionation, however, have yielded an active fraction which can reconstitute epitopes for the HLA-A2-restricted 660 TIL. This confirms the suitability of this general approach for the characterization of melanoma-specific antigens. Mass spectrometric analysis of the active fraction indicates the presence of at least two predominant peptides, each of which is a likely candidate for the melanoma-specific Ag and each of which is amenable to sequencing, synthesis and testing by cytotoxicity assays.

Although previous studies suggest that CTL recognize A2-restricted epitopes shared by the majority of melanomas, it is likely that multiple shared peptide-defined CTL epitopes on human melanoma cells may exist. Reconstitution experiments with 660 mel peptides may identify epitopes recognized by other HLA-A2-restricted melanoma-specific CTL. These shared epitopes may be derived by processing of a single or limited number of endogenous proteins, or perhaps may represent the product of distinct proteins. Determination of the amino acid sequences from the peptides and identification of the proteins from which they are derived will be important for the development of melanoma-specific diagnostics and therapeutics.

The overwhelming majority of patients with melanoma are Caucasians, 50% of whom express HLA-A2 molecules. HLA-A2-restricted epitopes, therefore, may provide the basis for significant diagnostic or therapeutic intervention. For example diagnosis based on host reactivity to these antigens may involve either immuno- or DNA-based assays. Both in vivo and in vitro applications may be considered. Therapeutic intervention may involve immunization of cancer patients with melanoma antigens for the purpose of stimulating melanoma-specific CTL and eradicating tumor cells.

Deposit of Biological Materials: The following cell line has been deposited with the American Type Culture Collection (ATCC), 12001 Parklawn Drive, Rockville, Maryland 20852 (USA) pursuant to the provisions of the Budapest Treaty.

Designation	Deposit Date	ATCC No.
660 mel	March 18, 1993	CRL 11304

Availability of the deposited strain is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The invention described herein draws on both published and unpublished work. By way of example, such work consists of scientific papers, pending patent applications, and patents. All of the work cited in this application is hereby incorporated by reference.

Also, the present invention is not to be considered limited in scope by the deposited cell line, since the deposited cell line is intended only to be illustrative of particular aspects of the invention.

The present invention has been described with reference to specific embodiments. However, this application is intended to cover those changes and substitutions which may be made by those skilled in the art without departing from the spirit and the scope of the appended claims.

We claim:

1. A polypeptide capable of being expressed on the surface of a tumor cell in association with HLA, said polypeptide when associated with HLA, is capable of rendering the tumor cell recognizable by a T cell.

2. The polypeptide of claim 1, wherein the tumor cell is a melanoma tumor cell.

3. The polypeptide of claim 1, wherein the HLA is HLA-A2.

4. The polypeptide of claim 1, wherein the T cell is a CTL or a T helper cell.

5. The polypeptide of claim 1, wherein the T cell is a TIL, the tumor cell is a melanoma tumor cell, and the HLA is HLA-A2.

6. The polypeptide of claim 7, wherein the TIL is 660 TIL, and the melanoma tumor cell is 660 mel.

7. The polypeptide of claim 5, wherein the polypeptide is selected from the group consisting of: mel Ag906 and mel Ag 1007.

8. A tumor-associated antigen capable of being expressed by a tumor cell, said tumor-associated antigen being obtainable from said tumor cell by a method comprising the steps of:

(a) obtaining an extract of the tumor cell;
(b) selecting HLA-associated antigens from the extract;
(c) purifying the HLA-associated antigens by means of HPLC;
(d) screening for HLA-associated antigens which are capable of associating with a non-tumor cell and thereby rendering it susceptible to lysis by a T cell or to cause the T cell to release cytokine or lymphokine in the presence of the non-tumor cell.

9. The tumor-associated antigen of claim 8, wherein in step (c), the HPLC fractionation comprises fractionating the sample according to hydrophobicity, using different reverse phase columns.

10. The tumor-associated antigen of claim 8, wherein in step (c), the HPLC fractionation comprises: C18, phenyl, C4, C8-polymeric fractionations.

11. The tumor-associated antigen of claim 10, wherein the tumor cell is a melanoma cell.

12. The tumor-associated antigen of claim 11, wherein the T cell is a TIL.

13. The tumor-associated antigen of claim 8, wherein the HLA is HLA-A2 and step (b) comprises the steps of :
(i) contacting the membrane extract with anti-HLA-A2 antibody such that the anti-HLA-A2 antibody binds to a complex of HLA-A2 and HLA-A2-associated antigen found in the membrane extract;
(ii) separating the complex of HLA-A2 and HLA-A2-associated antigen from the anti-HLA-A2 antibody; and
(iii) dissociating the HLA-A2-associated antigen from the complex of HLA-A2 and HLA-A2-associated antigen.

14. The tumor-associated antigen of claim 13, wherein in step (cc), the HPLC fractionation comprises: C18, phenyl, C4, C8-polymeric fractionations.

15. The tumor-associated antigen of claim 8, wherein step (a) comprises lysing the cell, releasing the polypeptide before it has associated with the HLA, and separating the polypeptide from the lysate by ultrafiltration.

16. The tumor-associated antigen of claim 8, wherein step (a) comprises acid extraction.

17. The tumor-associated antigen of claim 14, wherein the non-tumor cell is HLA-compatible with the tumor cell and T cell; and wherein the T cell is a tumor-specific T cell.

18. The tumor-associated antigen of claim 17, wherein the tumor cell is a melanoma cell, the tumor-specific T cell is a CTL.

19. A precursor polypeptide of the tumor-associated antigen of claim 8.

20. An end-product polypeptide of the tumor-associated antigen of claim 8.

21. A method for purifying a tumor-associated antigen from a tumor cell, said tumor cell is capable of being recognized by a T cell, said method comprises the steps of:

(a) obtaining a sample containing HLA-associated antigen from a membrane extract of the tumor cell;
(b) purifying HLA-associated antigen from the sample by HPLC fractionation; and
(c) screening for the HLA-associated antigen which is capable of associating with HLA on a non-tumor cell and causes the non-tumor cell to be to be susceptible to lysis by a T cell or to cause the T cell to release cytokine or lymphokine.

22. A method for diagnosing cancer in a patient, comprising the steps of:

(a) exposing a biological sample from the patient to an antibody raised against either a polypeptide which is capable of associating with HLA on a tumor cell to form a complex, such complex being recognizable by a T cell or the precursor protein from which that polypeptide is derived; and
(b) detecting the binding of the antibody to the sample.

23. A method for diagnosing cancer in a patient, comprising the steps of:
(a) exposing a biological sample from the patient to a nucleic acid probe encoding polypeptide which is capable of associating with HLA on a tumor cell and when such association is recognized by a T cell-specific to said tumor cell, said T cell would lyse said tumor cell or said T cell would release lymphokine or cytokine by said T cell; and
(b) detecting the binding of the nucleic acid sequence to the sample.

24. The method of claim 23, wherein the nucleic acid probe binds tumor cell genomic DNA or messenger RNA, and the nucleic acid probe is capable of differentiating between a wild type and an altered genomic DNA or messenger RNA.

25. A method for diagnosing cancer in a patient, comprising the steps of:

(a) exposing an individual to a composition comprising a polypeptide which is capable of associating with HLA on a tumor cell;
(b) observing the immunological reaction of the individual at the site of exposure.

26. A method for diagnosing cancer in a patient, comprising the steps of:

(a) exposing a sample of an individual which contains T cells, to a mixture of HLA and polypeptide which is capable of associating with HLA on a tumor cell ;
(b) observing the specific stimulation of T cells wherein the presence of such reaction indicates presence of tumor.

27. A method for treating a cancer patient comprising immunizing the patient with the polypeptide of claim 1 or the tumor-associated antigen of claim 8.

28. A method for immunizing a mammal against cancer, comprising immunizing the mammal with the polypeptide of claim 1 or the tumor-associated antigen of claim 8.

29. A pharmacological composition comprising the polypeptide of claim 1 or the tumor-associated antigen of claim 8.

30. An antibody directed against the polypeptide of claim 1 or the tumor-associated antigen of claim 8.

FIGURE 1

Specific Lysis of 660 Mel by Autologous 660 TIL

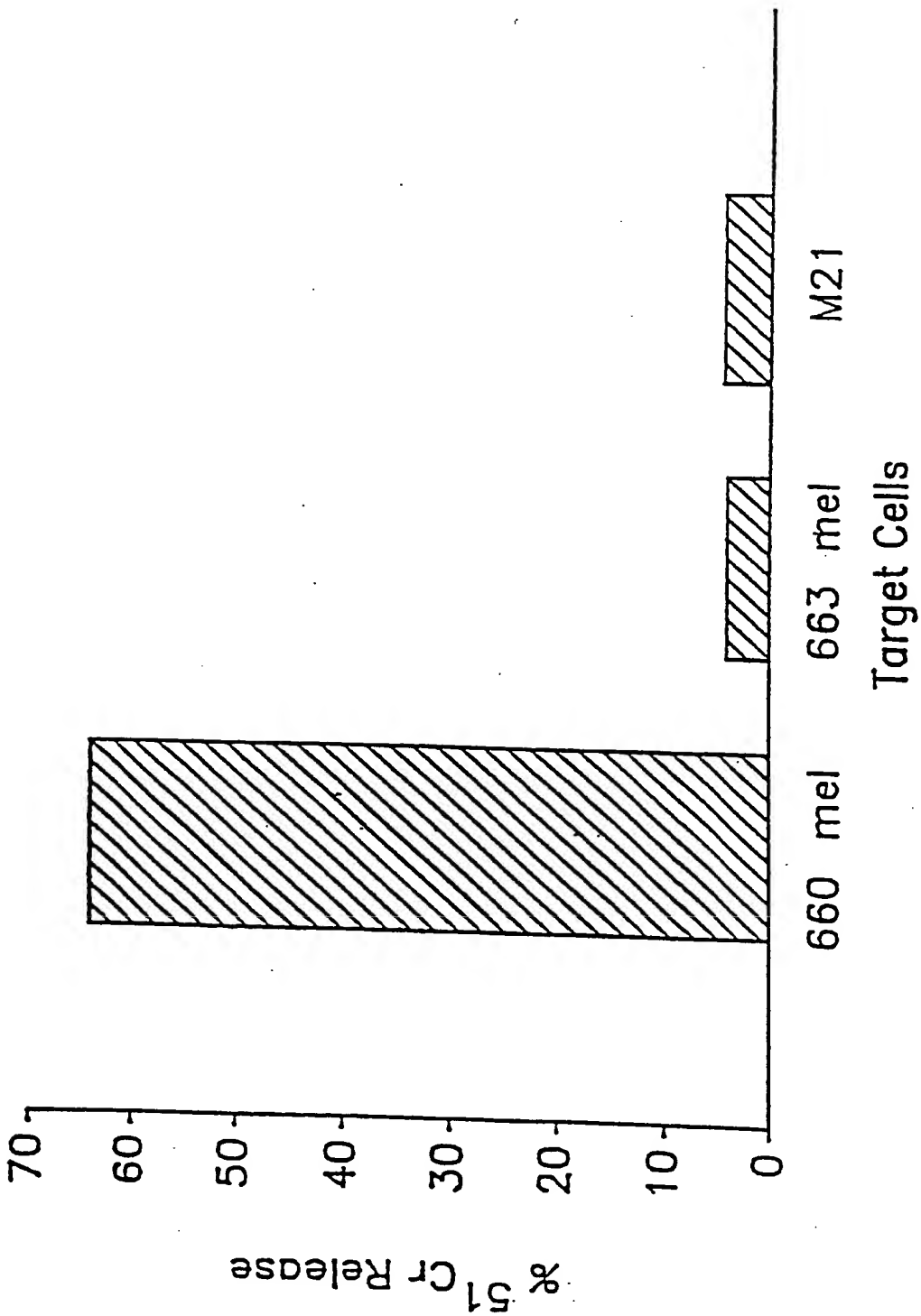


FIGURE 2

Increase of 660 TIL CTL Activity after Stimulation with Autologous Tumor Cells

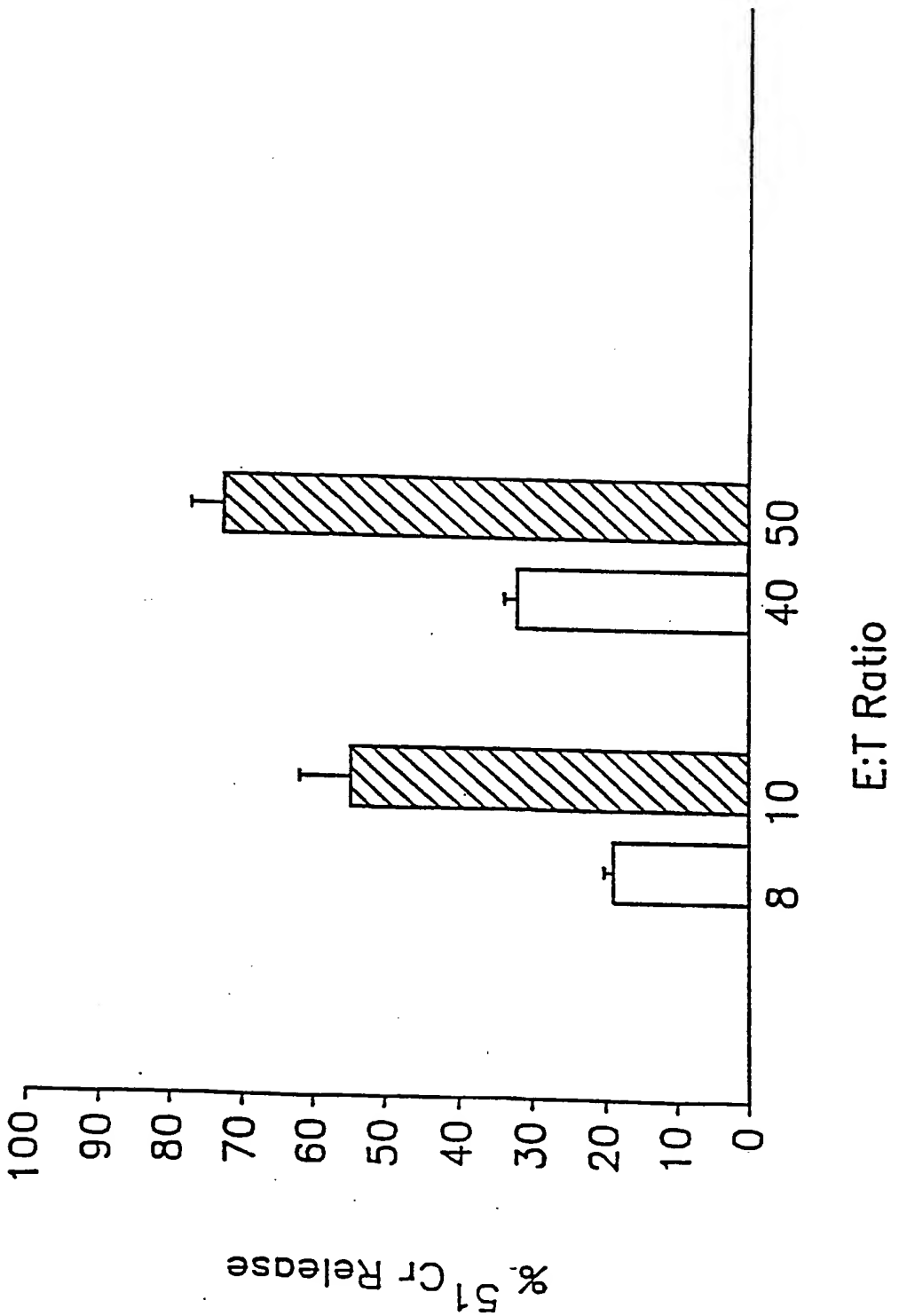


FIGURE 3

HLA A-2 Purification from 660 mel

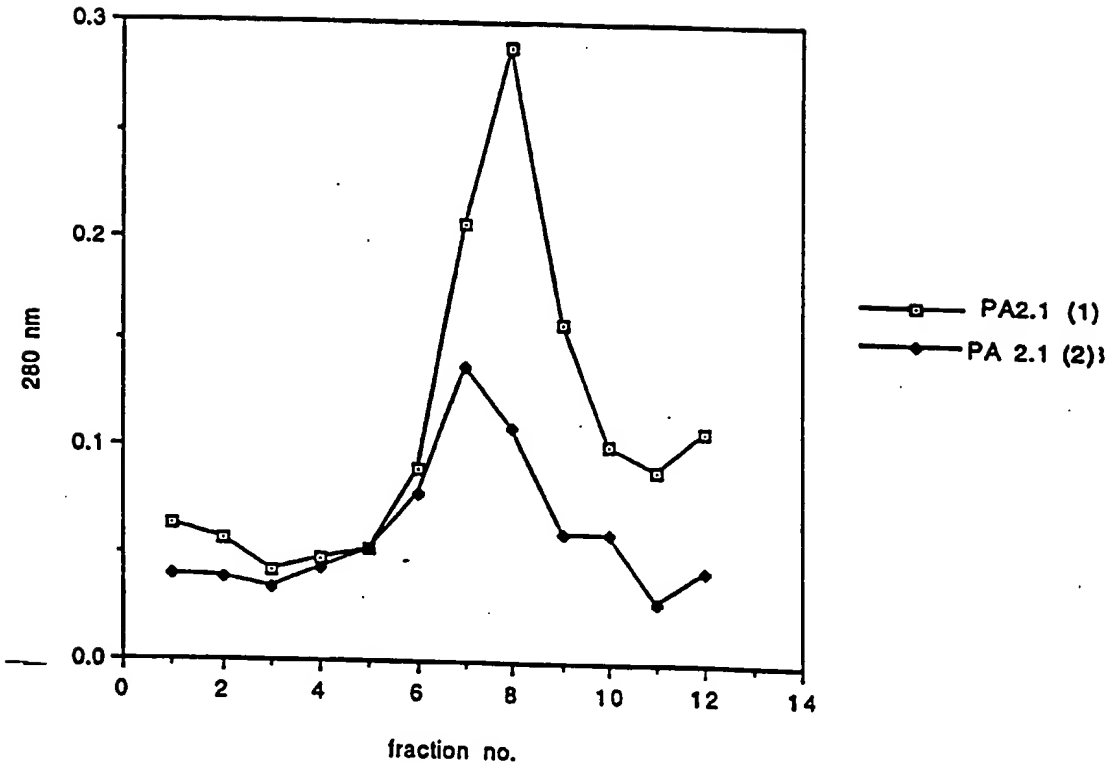


FIGURE 4

660 TIL CTL Assay: JY Target Cells + A2-Derived Peptides

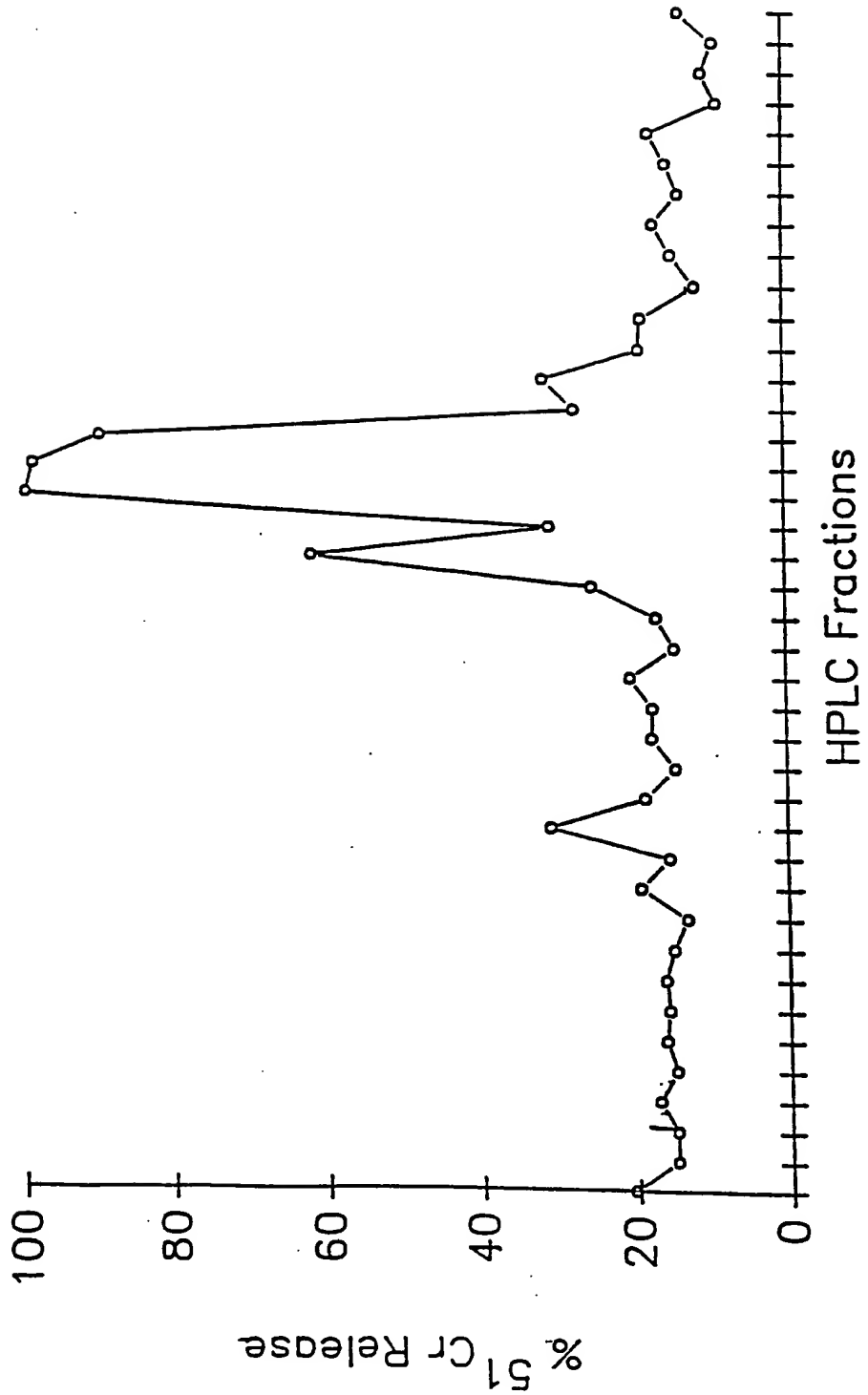


FIGURE 5

660 TIL CTL assay with 660 mel peptides from second HPLC column

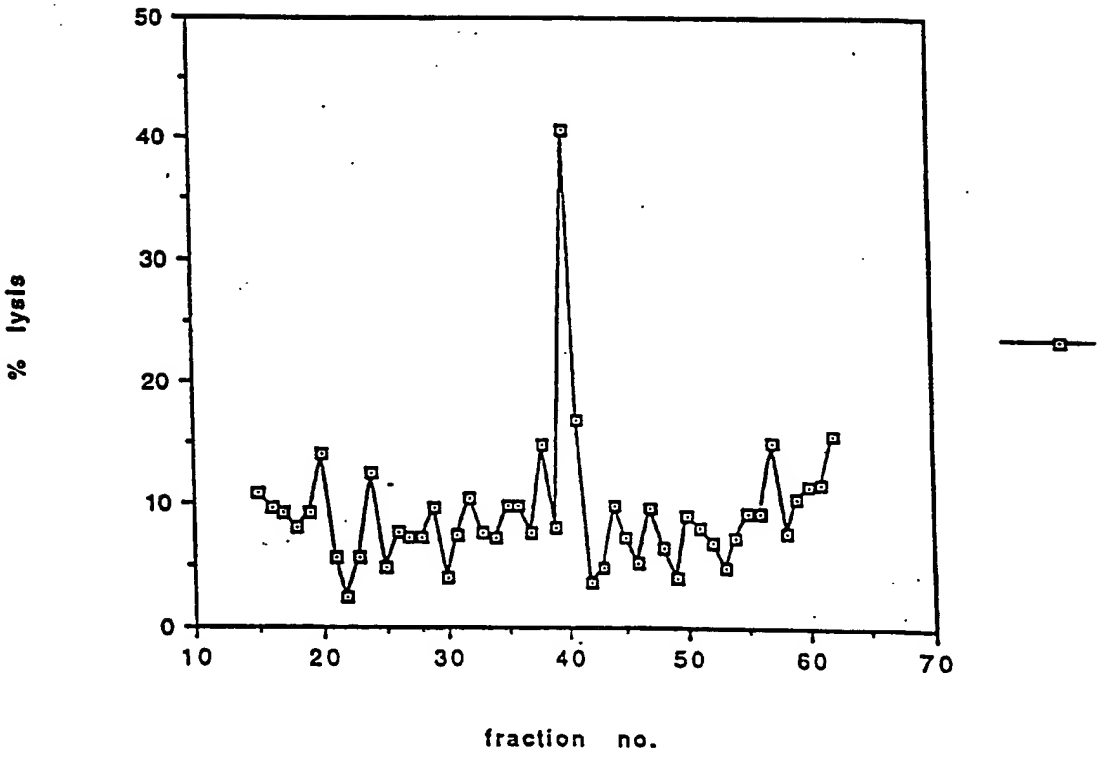


FIGURE 6

660 CTL assay with J-Y plus 660 mel peptides from third HPLC

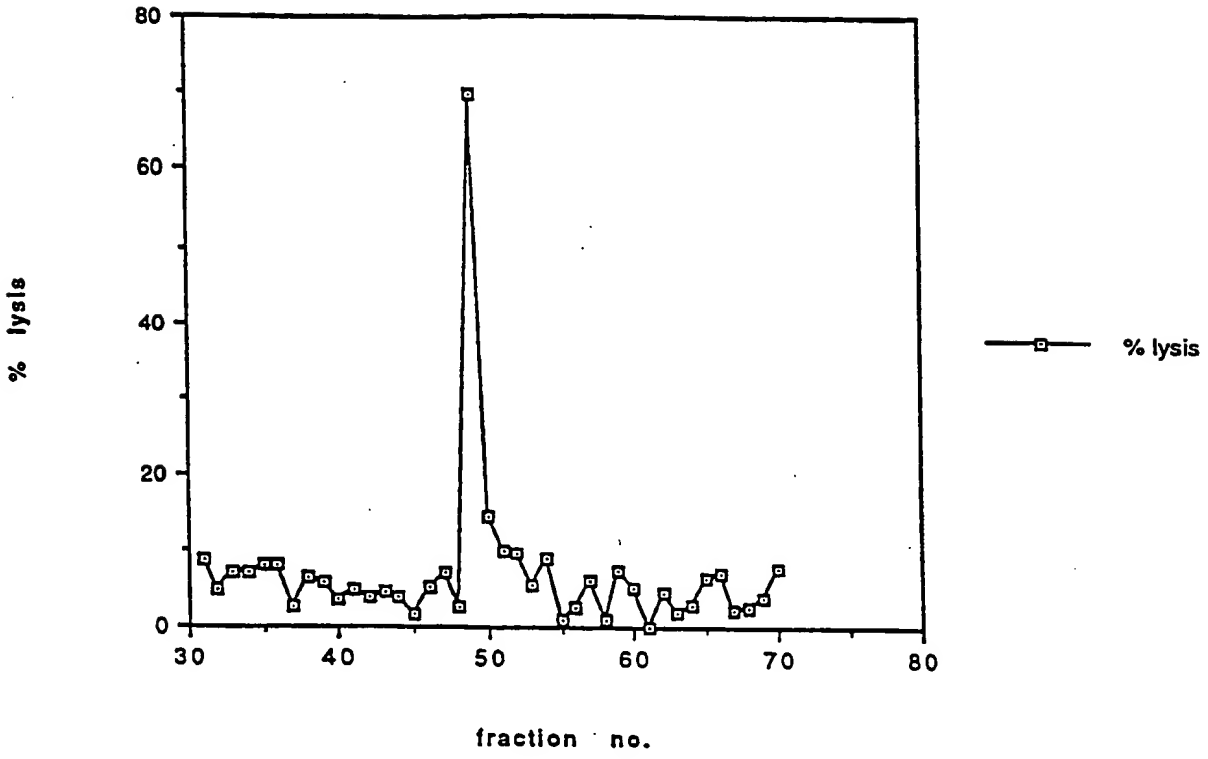
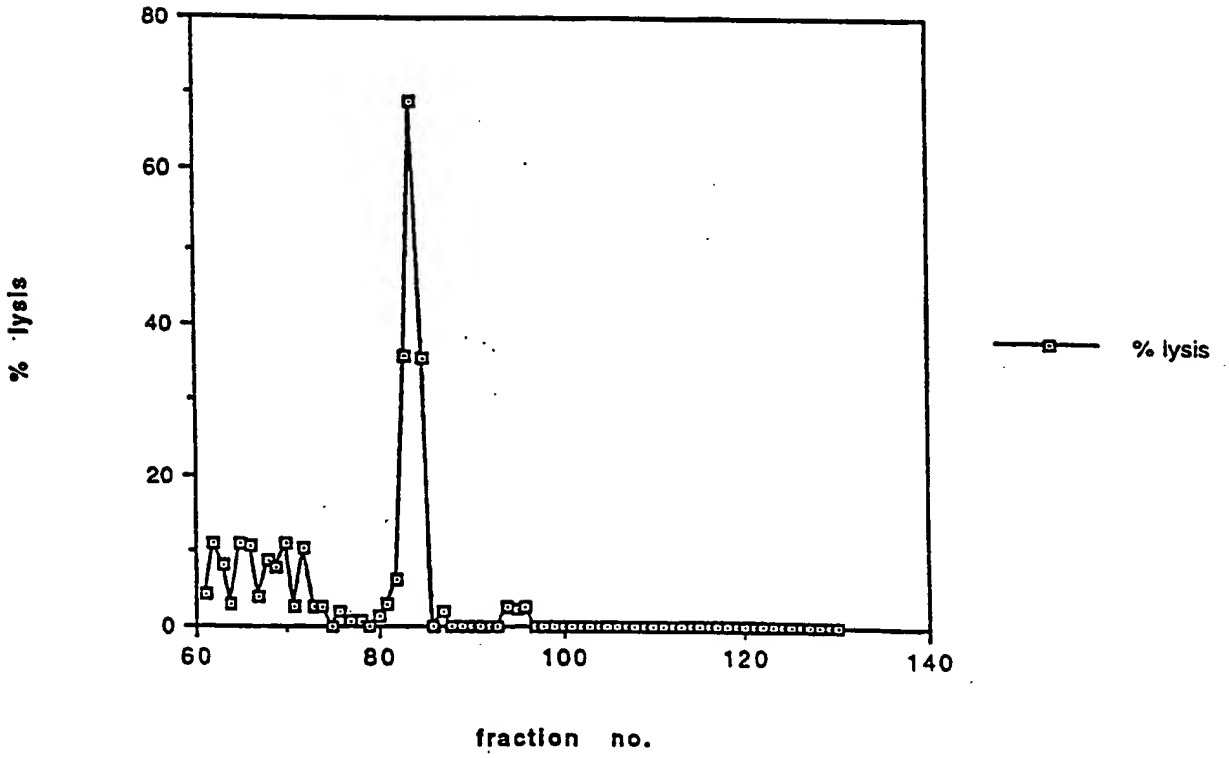


FIGURE 7

660 TIL CTL assay with J-Y plus 660 mel peptides from fourth HPLC



INTERNATIONAL SEARCH REPORT

Int. ional application No.
PCT/US94/03507

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 1/68; A61K 39/00, 39/395; C07K 13/00, 15/28
US CL : 435/6, 436/63, 64; 530/324, 350, 388.8, 389.7, 395
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.23; 436/63, 64; 530/324, 350, 388.8, 389.7, 395, 417

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS; DIALOG: MEDLINE, BIOSIS, CA, WORLD PATENTS
search terms: melanoma antigen, tumor antigen, mel Ag906, mel Ag1007, 660 mel

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Cellular Immunology, Volume 35, issued 1991, Reilly et al, "Increased Tumor-Specific CTL Activity in Human Tumor-Infiltrating Lymphocytes Stimulated with Autologous Tumor Lines", pages 526-533, especially pages 527 and 528.	1-20, 29 -------- 21-28, 30
X --- Y	Biochemica et Biophysica Acta, Volume 1080, issued 1991, Vlock et al, "Purification and Partial Characterization of a Shed 66 kDa Melanoma-Associated Antigen Identified by Autologous Antibody", pages 1-10, especially pages 2 and 3.	1-6, 8-20, 29, 30 ---------- 21-28

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 15 JUNE 1994	Date of mailing of the international search report JUN 27 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer CARLA MYERS Jill Warden for Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

Inte ional application No.
PCT/US94/03507

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Journal of Clinical Investigation, Volume 81, issued June 1988, Vlock et al, "Isolation and Partial Characterization of Melanoma-Associated Antigens Identified by Autologous Antibody," pages 1746-1751, especially pages 1747-1748.	1-6, 8-20, 29, 30 ---------- 21-28
X	Journal of Immunology, Volume 149, No. 4, issued 15 August 1992, Hayashi et al, "Molecular Cloning and Characterization of the Gene Encoding Mouse Melanoma Antigen by cDNA Library Transfection", pages 1223-1229, see entire document.	30
Y	Science, Volume 255, issued 06 March 1992, Hunt et al, "Characterization of Peptides Bound to the Class I MHC Molecule HLA-A2.1 by Mass Spectrometry", pages 1261-1263, see entire document.	21